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PRINCIPAL INVESTIGATOR: Zhou Wang, Ph.D.

CONTRACTING ORGANIZATION: Northwestern University
Evanston, Illinois 60208-1110

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) One critically important problem in prostate cancer research is to find new approaches to slow down the transition of prostate cancer from an androgen-dependent state to a lethal androgen-refractory state. Intermittent androgen ablation therapy may slow down the development of androgen refractory tumors because intermittent recovery of androgens can induce differentiation of prostatic epithelial cells. However, the advantage of inducing differentiation of prostate cancer cells by intermittent recovery of androgens is compromised by the disadvantage of androgenic induction of prostate cancer cell proliferation. The biologically most active androgen is dihydrotestosterone (DHT), which is converted from testosterone (T) by 5 α -reductase. Our recent studies showed that T is more potent than DHT in enhancing differentiation but weaker in stimulating proliferation, which led to our hypothesis that intermittent androgen ablation therapy can be enhanced by finasteride, an inhibitor of T to DHT conversion. To test our hypothesis in animal models, it is necessary to deliver exogenous T at physiologic levels and finasteride over a long period of time. We have worked out conditions to deliver T and finasteride in nude mice, which will allow us to test our hypothesis.				
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Introduction:

Conversion of T to DHT is essential for prostate development.

T and DHT are two major biologically active androgens (George and Wilson, 1994). T is synthesized in testis and then transported to target organs, such as the prostate, via blood circulation. T can be converted to DHT in the prostate by 5a-reductase (Bruchovsky and Wilson, 1968a; Bruchovsky and Wilson, 1968b). Both T and DHT bind to the same AR. DHT is more potent than T in activating promoters containing ARE, most likely due to the higher binding affinity of AR to DHT relative to that of T (Deslypere et al., 1992; Grino et al., 1990; Liao et al., 1973; Wilson and French, 1976). The conversion of T to DHT is necessary for normal prostate development because 5a-reductase inactivation prevents normal prostate development (Griffin et al., 1995; Walsh et al., 1974). It was thought that the conversion is merely an amplification step for androgen action (George, 1997). However, it cannot be ruled out that T and DHT have overlapping yet different biological functions *in vivo*. In fact, our recent studies suggest that T is more potent than DHT in inducing androgen-response genes during the regrowth of the rat ventral prostate (Dadras et al., 2001).

Androgens regulate homeostasis of prostate.

Androgens are required for the structural and functional integrity of the prostate (Bruchovsky et al., 1975). Androgen ablation by castration leads to rapid prostate regression via massive apoptosis (Colombel et al., 1992; English et al., 1989). On the other hand, androgen replacement stimulates rapid proliferation and differentiation of a regressed prostate until it reaches the normal size (Bruchovsky et al., 1975; Coffey et al., 1968). Androgen action in a regressed prostate is different from that in the fully-grown prostate because androgens do not stimulate proliferation in a fully-grown prostate (Table 1) (Bruchovsky et al., 1975). During the regrowth of a regressed prostate, androgens induce and then nullify proliferation, establish apoptotic potential while inhibiting apoptosis, and induce and maintain differentiation.

Table 1. The impact of androgen manipulation on the regressed prostate and the normal prostate.

Androgen	Regressed Prostate	Fully-Grown Prostate
+	Proliferation & Differentiation	No Significant Change
-	No Significant Change	Apoptosis & Dedifferentiation

+ represents androgen replacement and – represents androgen ablation or administration of anti-androgens. Differentiation is defined as the expression of prostate-specific markers. Dedifferentiation is defined as loss of prostate-specific marker expression.

Androgen action is intimately associated with prostate cancer pathogenesis.

Androgens are thought to play important roles in prostate cancer pathogenesis (Bosland, 1992; Carter and Coffey, 1990; Kozlowski and Grayhack, 1991). One of the risk factors for prostate cancer is the presence of the functional testis. Prostate cancer cells are derived from glandular epithelial cells and are initially androgen-dependent. Androgen ablation remains as the standard therapy for metastatic prostate cancer. Unfortunately, androgen ablation therapy is only

palliative and eventually patients relapse with androgen-refractory prostate cancer that is currently incurable (Kozlowski and Grayhack, 1991).

Development of androgen-refractory prostate cancer.

The mechanisms of prostate cancer progression from an androgen-dependent state to a lethal androgen-refractory state have been studied extensively. Mutations followed by clonal selection appears to be the mechanism of androgen-independent progression in several prostate cancer models, including the Dunning R3327 rat prostatic adenocarcinoma and LAPC9 human prostate cancer cells (Craft et al., 1999; Issacs et al., 1982). Another mechanism for androgen-independent progression involves adaptation. The androgen-independent progression of Shionogi mouse tumor and LNCaP human tumor involve the adaptation (Akakura et al., 1993; Bruchovsky et al., 1990; Kokontis et al., 1998; Umekita et al., 1996). It is possible that multiple mechanisms are involved in the development of androgen-refractory prostate cancer.

Intermittent androgen ablation therapy.

One urgent challenge in prostate cancer research is to develop new approaches to inhibit or to slowdown the development of androgen-refractory prostate cancer. Intermittent androgen ablation therapy was developed, attempting to delay the emergence of androgen-refractory prostate tumors relative to the continuous androgen ablation therapy. The rationale is that intermittent recovery of androgens can promote prostate cancer cell differentiation and enhance their dependence on androgens (Akakura et al., 1993; Sato et al., 1996). However, androgens are also proliferative to prostate cancer cells, which is undesirable in the therapy. The goal of our proposal is to increase the efficacy of intermittent androgen suppression by enhancing the differentiation effects while inhibiting the proliferative effects via finasteride administration.

Finasteride enhances the expression of many androgen-response genes during T-stimulated regrowth of the regressed prostate.

One interesting question in androgen action is whether or not the expression of androgen-response genes is differentially regulated by T and DHT. Finasteride, a 5 α -reductase inhibitor, had little or no effect on the expression of the surveyed androgen-response genes in testis-intact rats (Dadras et al., 2001). However, the induction of half of the surveyed androgen-response genes, including prostatein C3, adrenomedullin and calreticulin, are further enhanced by finasteride during T-stimulated regrowth of a regressed rat ventral prostate (Fig. 3) (Dadras et al., 2001). This unexpected observation suggests that T is more potent than DHT in inducing androgen-response genes in prostate regrowth.

Since finasteride only enhances androgen-response gene expression in a regressed prostate but not in a fully-grown prostate, finasteride is expected to enhance the expression of androgen-response genes in prostate tumor regrowth induced by intermittent recovery of androgens but not in prostate tumors untreated with androgen ablation therapy.

Body:

Task 1: Determine quantitatively the relative potency of T versus DHT in the induction of androgen-response genes during the prostate regrowth (Month 1-36).

- a. Animal manipulation and collecting prostatic tissue and serum samples.

- b. Measurement of serum and intraprostatic T and DHT.
- c. Measurement of DNA contents in the rat ventral prostate in the presence and absence of finasteride.
- d. Northern and Western blot analysis of androgen-response gene expressions in the rat ventral prostate in the presence or absence of finasteride.

Pilot studies on slow-releasing testosterone pellets.

According to our proposal, the first step in this project was to calibrate the T-pellets. We have contracted with Innovative Research of America (Sarasota, FL) to make 21 day slow-releasing pellets at different T dosages (Table 2). We used 3 rats for each condition in this pilot experiment.

	Testosterone (mg-21 pellets)	DHT (mg-21 pellets)	Finasteride	Number of Animals
Testosterone	0.1			3
	0.3			3
	0.5			3
	1			3
	2.5			3
	5			3
	15			3
Testosterone+Finasteride			40mg/kg/day	3
	0.1		40mg/kg/day	3
	0.3		40mg/kg/day	3
	0.5		40mg/kg/day	3
	1		40mg/kg/day	3
	2.5		40mg/kg/day	3
	5		40mg/kg/day	3
	15		40mg/kg/day	3
DHT			0.1	3
			0.3	3
			0.5	3
			1	3
			2.5	3
			5	3
			15	3
DHT+Finasteride			0.1	3
			0.3	3
			0.5	3
			1	3
			2.5	3
			5	3
			15	3
Castrate Only				
Normal Animals				
Total Number of Animals				94

Table 2. Experimental design of testing dose-response of androgen-stimulated prostate regrowth in the rat.

We have castrated the animals and left for 7 days, which allowed the prostate to regress. At day 7 after castration, the slow releasing T pellets or DHT pellets were injected subcutaneously with trocar. Finasteride treatment was started one day before androgen

replacement by daily subcutaneous injection of finasteride at 40 mg/kg. The animals were sacrificed two days after androgen replacement; serum samples were collected for testosterone measurement. Also, the ventral prostates were dissected out, weighed, and frozen for RNA extraction.

It appears that finasteride treatment slightly inhibited the regrowth of the prostate in 7-day castrated rats (Fig. 1). However, the differences were not statistically significant since only 3 animals were used in the study.

We have measured serum T levels in each animal. Results showed that serum T levels in animals received finasteride appeared lower than the T levels in the control animals (Fig. 2). This was not expected because previous publications from my lab and other labs showed that finasteride should not reduce serum T levels (Dadras et al., 2001). Finasteride also appeared to inhibit the DHT-induced prostate regrowth (Fig. 3). These observations raised our concerns whether the experiment was carried out properly or whether the slow-releasing pellets are compatible with finasteride treatment in our systems. Since we do not have the information on the materials responsible for slow-releasing, it was difficult for us to assess the compatibility of slow-releasing pellets with finasteride. We had additional problem with slow-releasing pellets in our mice experiment proposed in Specific Aim 2 (Fig. 5). Thus, we decided to first work out conditions to reproducibly deliver T, DHT, and finasteride.

Although the experiment was not as successful as planned, we did obtain some valuable information. It appears that the dosages for induction of half-maximum androgen-response gene expression are between 0.3 mg and 0.5 mg pellets (Fig. 4).

In task 2, we have worked out the approaches to deliver testosterone and finasteride in nude mice, which should also allow us to deliver T and finasteride in the rats. It is now feasible for us to accomplish the proposed Task 1.

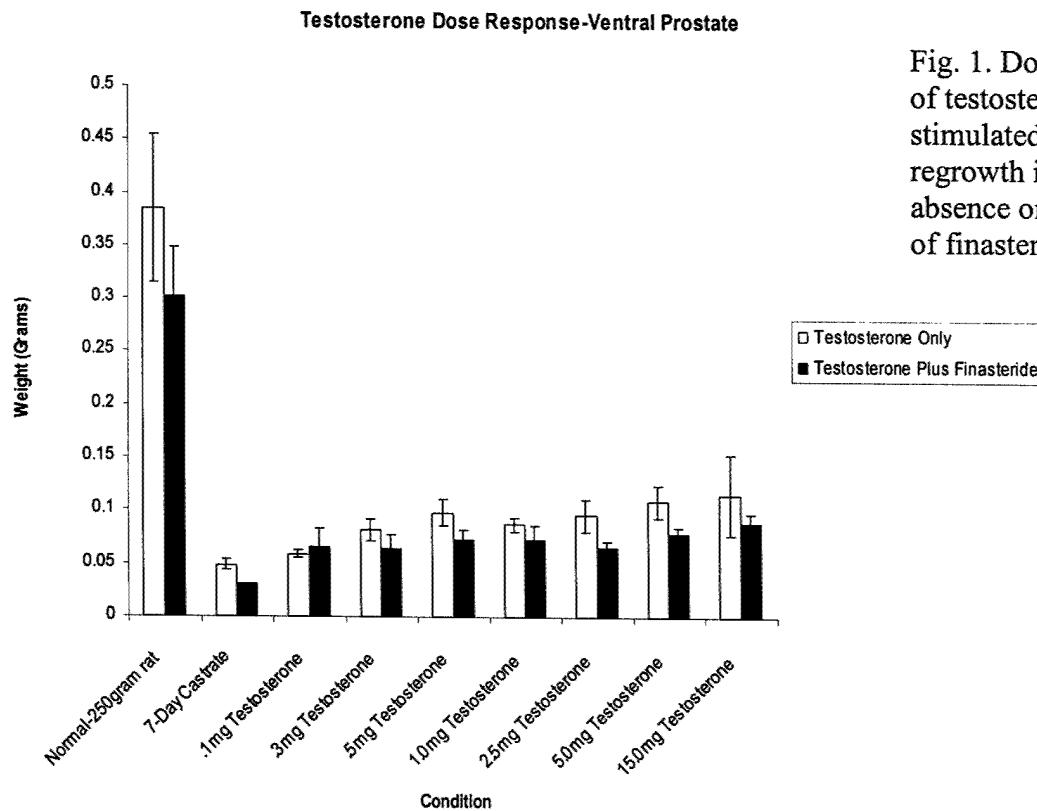


Fig. 1. Dose-response of testosterone-stimulated prostate regrowth in the absence or presence of finasteride.

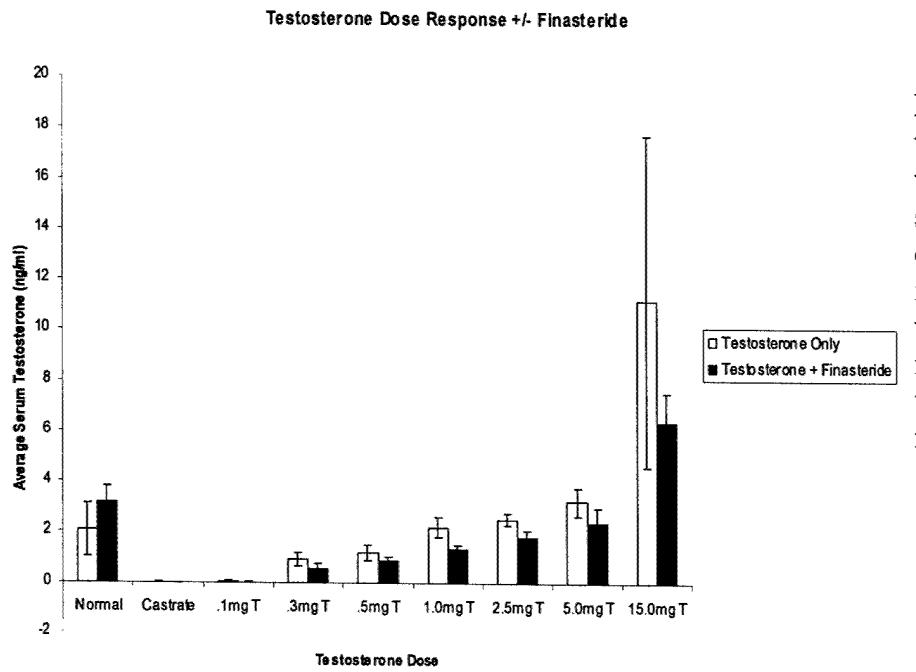


Fig. 2. Serum testosterone level in the dose-response study in the presence or absence of finasteride. The rats were implanted with indicated amount of testosterone in slow releasing pellets.

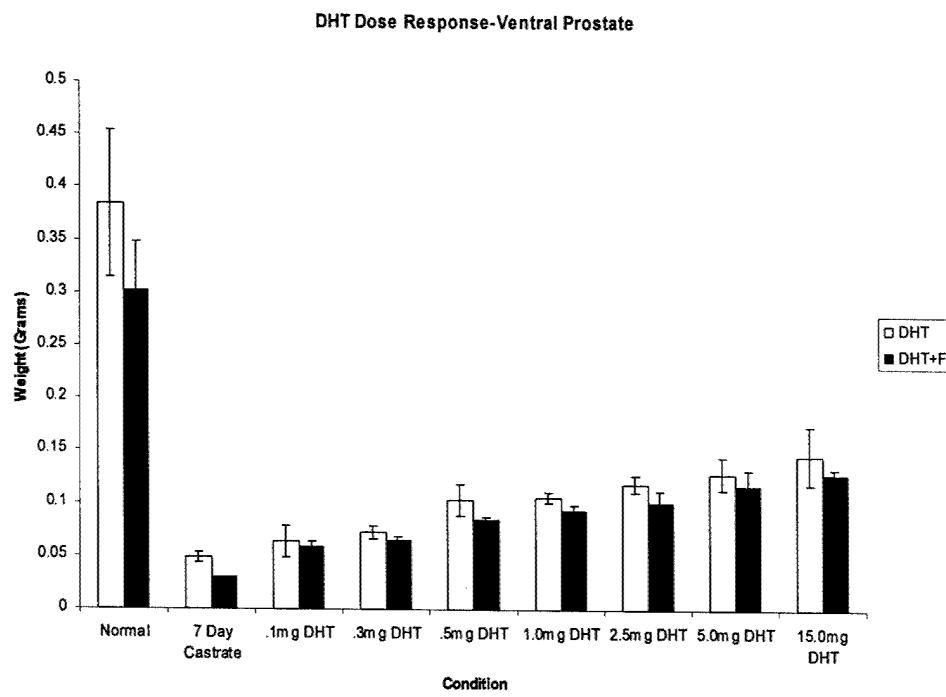


Fig. 3. Dose-response of DHT-stimulated prostate regrowth in the absence or presence of finasteride. The rats were implanted with indicated amount of DHT in slow releasing pellets.

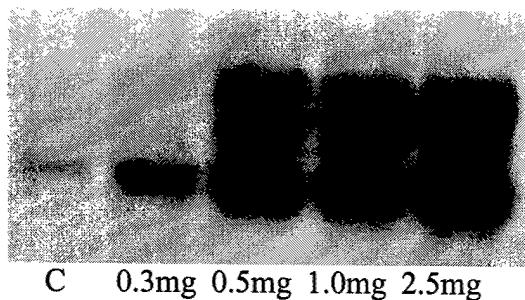


Fig. 4. Dose-response of spermidine synthase expression in the rat ventral prostate. Northern blot analysis was carried out. The total RNA was isolated from the ventral prostate in castrated rats treated with indicated amount of T in slow releasing pellet.

Task 2: Test the effect of finasteride on intermittent androgen ablation therapy of xenograft androgen-sensitive prostate tumors in nude mice (Months 1-36).

- Establish Shionogi and LNCaP androgen-sensitive tumor models in nude mice.
- Determine the impact of finasteride on intermittent androgen ablation in Shionogi model by collecting tumor specimens and serum samples for analysis.
- Determine the impact of finasteride on the time required to establish androgen-independent PSA expression in LNCaP tumor model undergoing intermittent androgen suppression.

Pilot studies on slow-releasing of finasteride and physiological doses of testosterone in nude mice.

Daily subcutaneous injection of testosterone and finasteride in nude mice over a period of several months is not practical. Subcutaneous injection of large volumes of sesame oil and propylene glycol, vehicles for finasteride and T, respectively, are likely to be detrimental to the host animals. Thus, it is necessary for us to work out a reliable way to deliver testosterone and finasteride over a long period of time, without of daily injection.

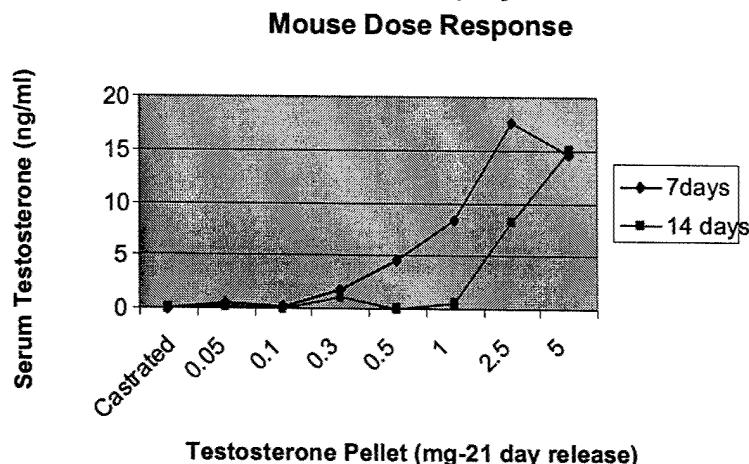


Fig. 5. Serum T level in castrated nude mice implanted with indicated mg of T in slow-releasing pellets 7 and 14 days after implantation.

We first tried to determine what doses of 21-day testosterone pellet is adequate for achieving physiological T levels. In our pilot experiment, we have tested 7 doses of 21-day testosterone pellets: 0.05mg, 0.1mg, 0.3mg, 0.5mg, 1.0mg, 2.5mg, and 5.0mg. A total of 14 male nude mice were used in our experiment with 2 mice in each group. The mice were castrated for 7 days and then injected subcutaneously with slow-releasing T pellets by trocar. One of the two mice in each group was sacrificed 7 days after T pellet injection and the other sacrificed 14 days after the T replacement. Serum T levels were determined for each animals. The results in Fig. 5 showed that serum T was detectable 7 days after injection of ≥ 0.3 mg T. However, after 14 days of T replacement, the serum T was only detectable when 2.5 mg and 5 mg T were injected. This observation suggested that the low T dosage 21-day release pellets lasted less than 14 days. This pilot experiment indicated that we could not rely on these commercially available slow releasing pellets.

As an alternative, we performed pilot experiments using silastic tubing to 1) achieve physiologically relevant levels of testosterone in nude mice; and 2) determine the dosage of finasteride implants to appropriately decrease serum or intraprostatic DHT.

In our first pilot experiment, silastic tubing (inside diameter 1.98 mm, outside diameter 3.18 mm, wall thickness 0.61 mm) were placed subcutaneously with either testosterone or finasteride using the following design:

Group 1 (3 mice) noncastrate controls.

Group 2 (3 castrate mice) with 1 mm testosterone implants.

Group 3 (3 castrate mice) with 1 mm testosterone implants and 1 cm finasteride implants.

Group 4 (3 castrate mice) with 2mm testosterone implants.

Group 5 (3 castrate mice) with 2 mm testosterone implants and 1 cm finasteride implants.

Group 6 (2 castrate mice- 1 died during induction of anesthesia) with 1 mm testosterone implants and two 1 cm finasteride implants.

Group 7 (3 castrate mice) with 2 mm testosterone implants and two 1 cm finasteride implants.

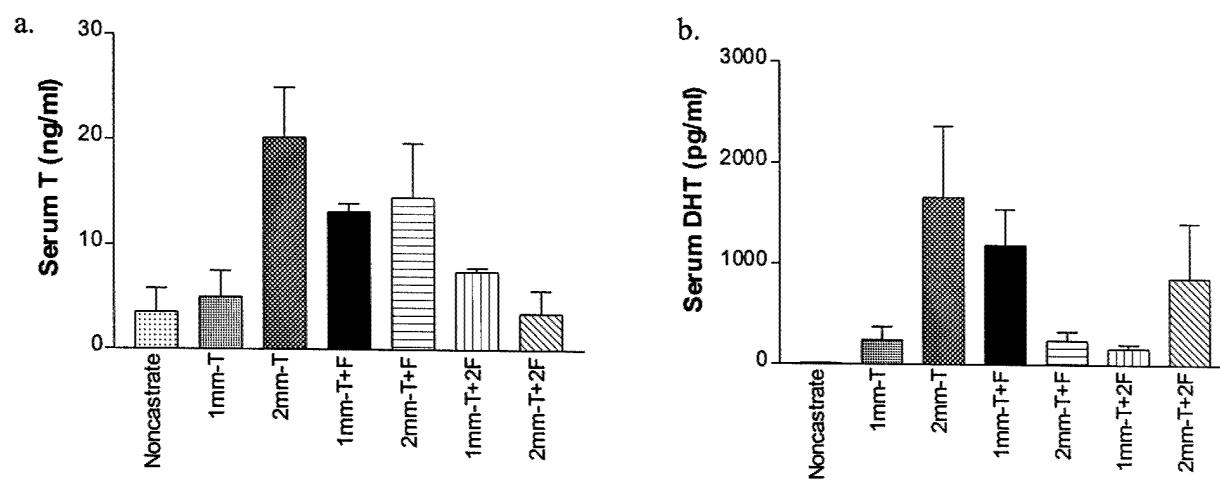


Fig. 6. a. Serum T levels in noncastrate nude mice and castrated nude mice implanted with indicated amount of testosterone (T) and finasteride (F). b. Serum DHT levels in noncastrate nude mice and castrated nude mice implanted with indicated amount of testosterone (T) and finasteride (F).

Mice were sacrificed 2 weeks after the implantation and serum was drawn by cardiac puncture. Serum testosterone levels and DHT levels were measured by radioimmunoassay. In this set of experiment, we did not attempt to measure intraprostatic T and DHT levels in individual animal because we thought that the mouse prostate is too small to provide enough material for measurement.

From the serum T and DHT measurement (Fig. 6a), it seems that the 1 mm T delivery was not consistent while 2 mm T caused supraphysiological serum T level. It is difficult to determine whether finasteride inhibited T to DHT conversion in this experiment (Fig. 6b), which may be due to the presence of finasteride-insensitive type I 5a-reductase. A major weakness of this experiment was that we did not measure intraprostatic T and DHT levels.

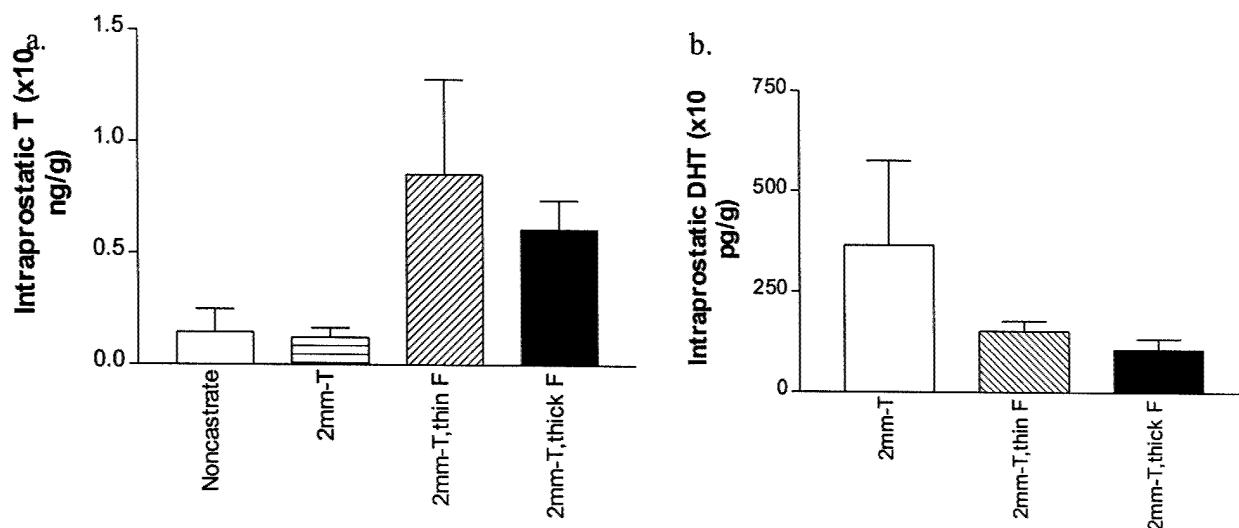


Fig. 7. a. Intraprostatic T levels in noncastrate nude mice and castrated nude mice implanted with indicated amount of testosterone (T) and finasteride (F). b. Intraprostatic DHT levels in castrated nude mice implanted with indicated amount of testosterone (T) and finasteride (F).

From the results of the above experiment, we conclude that we need to deliver less T over a longer period of time and to deliver more finasteride to achieve effective inhibition of T to DHT conversion. To accomplish the above objectives, we have used different types of silastic tubings. Thicker-walled silicone tubing was used (inside diameter 3.18 mm, outside diameter 6.35 mm, wall 1.59 mm) to deliver testosterone. Two different types of tubing were used to deliver finasteride. The first ("thin pellets") had a very thin outer wall (1.47 mm inside diameter, 1.96 mm outside diameter, 0.23 cm wall). The second ("thick pellets") had a greater inside diameter in order to increase the surface area of finasteride exposed to the mouse (3.35 mm inside diameter, 4.65 outside diameter, 0.66 mm wall).

Group 1 were three noncastrate controls, and the remaining groups each had four castrate mice.

Group 2 were implanted with 2 mm testosterone implants.

Group 3 had 2 mm testosterone implants along with 1 cm finasteride "thin tubing."

Group 4 had 2 mm testosterone implants along with 1 cm finasteride "thick tubing."

Mice were sacrificed 2 weeks after implantation, at which time serum was drawn by cardiac puncture and the ventral prostate was dissected and frozen in liquid nitrogen. Both were stored at -80 degrees. Testosterone levels were measured by radioimmunoassay. DHT levels were initially measured by a direct RIA kit, which proved to be not accurate. We re-measured intraprostatic DHT levels from the samples that we had left. We were able to measure intraprostatic T and DHT in most of the animals using a modified protocol.

To determine whether finasteride is effective in mouse prostate, we needed to first work out a method to measure mouse prostatic T and DHT. We have modified the method we worked out previous for measuring rat prostatic T and DHT (Dadras et al., 2001). We were able to measure intraprostate T and DHT in the mouse prostate individually (Fig. 7). Our studies indicated that finasteride significantly inhibited the conversion of T to DHT in the prostate in the second pilot experiment. As expected, the presence of finasteride enhanced the intraprostatic T level and reduced the intraprostatic DHT levels. We have also observed a significant reduction in the size of seminal vesicles in mice implanted with finasteride relative to the controls, further indicating the effectiveness of finasteride tubing in nude mice. Because the release from the "thick" tubing was slow, there was a lot of T and finasteride left in these tubings after two weeks implantation.

Having demonstrated the effectiveness of finasteride tubing, we are in the process of establishing LNCaP xenograft tumors to determine whether finasteride coupled intermittent can delay androgen-independence progression of prostate xenograft tumors. We have showed that we are able to establish LNCaP xenograft tumors over 70% take rate in our current experiment (results not shown).

Task 3: Determine the effect of finasteride on the expression of androgen-response genes in LNCaP tumors during intermittent androgen ablation therapy (Month 24-36).

- a. Collect LNCaP tumor specimens and serum samples from nude mice.
- b. Determine the expression of androgen-response genes, adrenomedullin, calreticulin and PSA, in LNCaP tumors.
- c. Analysis of the collected data and prepare the final report for the proposal.

N/A.

Key Research Accomplishments:

1. Delivery of exogenous T at physiologic levels over a prolonged period in nude mice.

A prerequisite of the proposed project is to deliver physiologic doses of T over a long period in nude mice. In our experiment, we were unable to deliver T over prolonged period using slow releasing T pellets prepared by Innovative Research of America, FL. Fortunately, we were able to overcome this technical challenge and work out a condition using the "Thick"

silastic tubing to deliver T in nude mice. This knowledge could potentially benefit other investigators.

2. Delivery of finasteride over a prolonged period in nude mice.

Daily subcutaneous injection of finasteride dissolved in large volume of sesame oil over a long period of time would be painful to the host and may cause adverse effects. Thus, it is necessary for us to avoid daily subcutaneous injection. We have tested three different types of silastic tubing and find one of them would allow us to deliver finasteride over a long period of time. This method permits us to explore the effect of finasteride in intermittent androgen ablation therapy of prostate cancer in xenograft tumor models.

3. Measurement of intraprostatic T and DHT in individual nude mouse.

To demonstrate that finasteride inhibits T to DHT conversion in mouse prostate, it is necessary for us to measure the T and DHT in the mouse prostate, which is very small. Using a modified radioimmunoassay, we were able to measure T and DHT in individual mouse prostate, which allowed us to show the finasteride inhibition of T to DHT conversion in the mouse prostate.

Reportable Outcomes:

None.

Conclusions:

Our proposed research requires the ability to deliver appropriate doses of finasteride and T over a prolonged period in nude mice. In the past year, we have encountered difficulties because the commercially available slow releasing pellets did not work in our system. After some investigation, we decided to focus on silastic tubing for slow delivery of T and finasteride. After multiple tests, we now resolved these critical technical problems and will be able to achieve the proposed objectives.

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Appendices:

None.